The Comparative Viability of Seeds, Fungi and Bacteria When Subjected to Various Chemical Agents

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The comparative Viability of Seeds, Fungi and Bacteria when subjected to various chemical Agents.

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With 1 Textfigure.

The following work was undertaken because of the lack of conclusive evidence that it is possible to obtain seeds for experimental purposes, free from contaminating organisms.

All of the work was done in the botanical laboratory of the University of Michigan, under the supervision of Professor Newcombe, to whom I wish to acknowledge my indebtedness. For valuable aid and helpful suggestions on the mycological side of the problem, I am greatly indebted to Prof. J. B. Pollock, also of this laboratory. To Dr. F. G. Novy of the medical department, I am indebted for some valuable suggestions on the bacteriological side, also to his assistant, Mr. W. A. Perkins, for valuable practical aid.

Historical.

Very little has been done to study the comparative viability of seeds, fungi and bacteria when treated with different disinfecting agents. It is only within recent years that any work having that end in view has been done. The attempted object is to obtain good seedlings, free from bacteria and fungi,

for experimental purposes. The earlier efforts along the line of sterilization were to sterilize the hands, articles of clothing, rooms, etc. For the hands, mercuric chloride mainly was used. Some valuable suggestions as to its antiseptic action may be obtained from the work of Fürbringer and Freyhan (1897) and of Danielsohn and Hess (1902). The work along these lines is suggestive of the possibility of obtaining seedlings, free from bacteria and fungi, for experimental purposes.

After sulphur was found to be, not valueless, but inadequate, for room sterilization, attention was more and more directed to formaldehyde. It is conceded quite generally that formaldehyde is valuable only as a surface disinfectant. Special emphasis is laid on this point by Novy and Waite (1898) in their paper on „Disinfection of Rooms." They found that dried or covered infectious material was not necessarily killed even after twenty hours exposure to the gas. Müller (1901) also came to the conclusion that only exposed bacteria are killed; further, that these must be vegetating forms. Rubner and Peerenboom (1899) found that horizontal surfaces might be sterilized by means of formaldehyde, while vertical surfaces were not in the least affected. This they ascribe to a condensation of the formaldehyde gas, causing it to settle in disinfecting quantities on the horizontal surfaces, while the vertical surfaces receive no such deposit.

The work done on seeds with formaldehyde is, as a general rule, very unsatisfactory. Kehler (1904) did some work in that direction, but he found that his seeds were more sensitive to the action of formaldehde than the spores of either bacteria or fungi, when its action went deeper than the mere surface, as is absolutely essential if one is to obtain sterile seeds. The work of Werner (1904) indicates that a stronger solution of formaldehyde than seeds can bear is necessary to kill even exposed bacterial spores. Chester and Brown (1905), studying the action of formaldehyde in milk, found that it took a one-eighth percent solution over five days to kill Bacillus subtilis. Bosc (1896) found that it took five hours to kill pathogenic germs on cloth well exposed to formaldehyde gas. Sternberg (1901) and Park (1905) lay greater emphasis than any of the others on the chemical action of formaldehyde in disinfection. Morse (1907) found that formaldehyde will kill Phytophthora infestans, a parasitic fungus, on seed potatoes, without injuring the potatoes.

The best results to date in sterilization were obtained by the use of mercuric chloride. Kehler (1904) found that copper sulphate killed seeds so quickly that it was valueless as a disinfecting agent. He, however, obtained excellent results with mercuric chloride. Miyajima (1897) found that a 0,3 per cent solution of mercuric chloride killed the seeds of Zea mays, Pisum sativum and Vicia faba. The harm was done by overexposure, the seeds being exposed for six hours or more. Czapek (1896) found that he could obtain sterile seeds of Zea mays by polishing the dry seeds with a stiff brush till no more particles came off, then cleaning them, thoroughly with a brush, soap and warm, sterile, distilled water, and finally dipping them for two or three minutes in a 1 per cent solution of mercuric chloride, and then rinsing them once in sterile distilled water. This treatment, he claims, was sufficient to kill the fungus hyphae growing over the aleurone layer, but not into it.

Freeman (1904), working also on Lolium temulentum, treated the seeds with a 1 per cent solution of mercuric chloride, but found

only some of the seeds to be free from the fungus. Steward (1908) found that the seeds of Zea mays when subjected for one-half to three-quarters of an hour to a 0,5 per cent solution of mercuric chloride were still sterile at the end of fourteen or sixteen days. Paul and Krönig (1896) found that a two percent solution of mercuric chloride was unable in twenty-five minutes to destroy the spores of Bacillus anthracis in a suspension dried on the surface of tare-garnets. Behring (1888) attributed the failure of mercuric chloride in sterilization to the formation of mercuric albuminates. Nelson (1907), treating potatoes for Oospora scabies, found that a 1 percent solution of mercuric chloride killed the parasite without injuring the potatoes. Some interesting work on the inhibition of bacteria was done by Paul (1901). He found that a 1 to 1,000 000 solution of mercuric chloride kept them in check without killing them. Geppert (1889) found that a suspension of Bacillus anthracis dried on silk threads was prevented from developing by treating for ten minutes with a 0,1 percent solution of mercuric chloride. However, after having been treated for a half-hour with a 0,1 percent solution the organisms were caused to develop by precipitating the mercury with ammonium-sulphate. Eriksson (1905) attempts to explain the overwintering and consequently the persistence of wheat-rust under treatment by what he calls the Mycoplasm theory. He assumes that part of the protoplasm in the cells belongs to the host but that part of it belongs to the fungus and is to all apperance dormant. This theory appears rather fanciful in the light of the researches of Stoward (1908), Hannig (1908), Bolley and Pritchard (1905) and others.

Technique.

The work laid in this study was to test the action of the following agents: cleaning fluid[1]), mercuric chloride, hydrogen peroxide, potassium dichromate, ammonium persulphate, bromine water (common), and formaldehyde gas, on the seeds of Lupinus albus, Pisum sativum, Triticum vulgare, Hordeum vulgare, Zea mays and Sinapis alba.

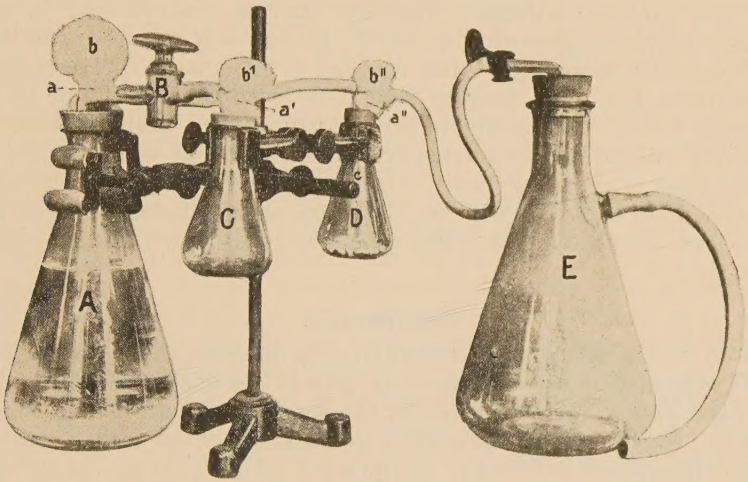
The work can be convently divided into two parts. The first part has to do mainly with the seeds, the second mainly with the fungi and bacteria.

The first thing to be done is to determine the highest concentration and the longest period of exposure in each case which will still permit one to obtain a fair percentage of normal seedlings, leaving out of consideration for the time being the effect of the disinfectant on the adhering fungi and bacteria. A great deal of unnecessary and tedious work would be involved in determining the effect of the disinfectant on the fungi and bacteria adhering to the seeds while determining the effect on the germination of the seeds. The germination tests were carried on during the early fall when the laboratory had a normal temperature day and night of 22° C. This temperature was fairly constant. Lots of twenty-five seeds each were exposed for different lengths of time to different concentrations of each of the disinfectants, and, after thorough washing, were placed in a Geneva germinator kept at room temperature. They were left for four or five days and were then compared with the controls. These controls were treated as nearly as possible in a

[1]) Cleaning fluid = H_2SO_4 sp. gr. 1.83 saturated with K_2CrO_7.

manner similar to the experimental seeds, except that no disinfectant was used.

After the highest point of both exposure and concentration had been determined for each kind of seeds in the different disinfectants, each kind of seeds thus treated was then tested to determine whether bacteria and fungi could endure this treatment as well as the seeds. For determining the latter point, an apparatus was constructed, the idea for which was obtained from Kehlers (1904) paper. It differs from Kehlers apparatus in that the seeds, after they are once placed in the disinfectant, are not exposed to contamination in any form till after a two weeks incubation. Kehler transferred his seeds by means of sterile forceps from the vessel in which they had been treated to the flask of culture medium.



My apparatus (see te t-figure) is constructed as follows: There is a large flask, A, which contains distilled water and is connected with a three-arm glass cock, B. Flask A also has a glass tube, a, with a flange worked on the end. This tube projects into the flask through the rubber stopper. Over the flanged end of the tube is securely fastened a cap of cotton, b, to filter the air as it enters the flask when the water is drawn out of the flask. To the tube on the opposite side of cock B, is attached a small flask C. This flask has also an upright flanged tube, a′, with a cotton filter b′. To the third tube of cock A is attached another small flask D. In addition to the flanged tube, a″, with its cotton filter b″, it has a third tube, c, entering it. This tube is drawn to a point, dips to the bottom of the flask, being bent so as to end in the angle between the side wall and the bottom of the flask. Tube c is connected with cistern E, which in turn is connected with an aspirator. Flasks A, C and D are supported on a low ringstand.

The apparatus is operated as follows: Flask A is filled with distilled water; into flask C is put 25 ccm of agar medium; and E is disconnected after the rubber tube between D and E has been closed by means of a clamp. The whole apparatus (except the cistern) is then placed in the autoclave and sterilized. After autoclaving, flask C, while still attached to the ringstand, is introduced through a slot into a large cardboard cylinder.

Around the arm supperting flask C is packed sufficient cotton to close the opening in the cylinder. E is again connected with the rest of the apparatus. When the apparatus has cooled sufficiently, the rubber stopper is removed from flask D, the seeds and the desired disinfectant are quickly introduced, and the flask is again closed tightly. Flask D is then thoroughly shaken, so that the disinfectant may come in intimate contact with the walls of the flask, the tube and the stopper. This is to destroy any spores that may have happened to enter when the flask was opened to admit the seeds and disinfectant. When the seeds have been in the disinfectant the required length of time, the disinfectant is drawn off into the cistern E, by means of tube c. The last drops can readily be removed by tilting flask D. Water is then drawn through cock B from flask A, into flask D, upon the seeds. This is done by exhausting the air in cistern E. To prevent the air from entering flask D during this process, a rubber finger-cot can be drawn over b, or the finger may simply be pressed down on it. In that way the seeds can be washed as frequently as desired. After washing them, some of the agar medium is drawn from flask C by turning cock B so as to connect C and D. Agar solidifies at 42° C. It is therefore kept at about 45° C until needed. That temperature will not harm the fungi or bacteria that may adhere to the seeds. Sufficient agar to cover the seeds is introduced. The rubber tubes between D and C and between D and E are closed by means of clamps. The rubber tubes are cut beyond the clamps and flask D is ready be set aside to incubate.

Experimental.

In all of the following germination experiments, all of the seeds were carefully inspected so as to obtain only perfect specimens. In all the germination experiments, each lot consisted of twenty-five seeds. The germination percentage, under ordinary laboratory conditions, of the seeds in the following experiments was as follows: Lupinus albus 96 per cent, Pisum sativum 96 per cent, Triticum vulgare 96 per cent, Hordeum vulgare 92 per cent, Zea Mays 100 per cent, and Sinapis alba 96 per cent. The first agent used in treating the seeds was cleaning fluid ($H_2SO_4 + K_2Cr_2O_7$). As far as I have been able to ascertain it has not been employed for the purpose before. It occured to me that cleaning fluid might be a very desirable agent to use since it penetrates no farther than it thoroughly oxidizes. Thus, by timing its action, we can destroy as much of the seed-coat as is safe under any given condition, and also the adhering fungus and bacteria spores, even such fungus hyphae as may have penetrated part way into the seed-coat. From what I knew of the properties of cleaning fluid, I had no idea that any seeds were so resistant to its action. For instance, Lupinus albus immersed in it for five hours and fifteen minutes still gave 52 per cent of good seedlings. Up to about two hours immersion Lupinus albus gave, in practically every case, about 100 per cent of good seedlings. Pisum sativum was also more resistant than was expected. After six minutes exposure to this fluid, 88 per cent of good seedlings were obtained; and after forty-five minutes immersion, the yield was still 76 per cent. Triticum vulgare and Hordeum vulgare were found to be rather sensitive to the action of cleaning fluid. After seven minutes exposure, the former yielded only 28 per cent of good seedlings; while after ten minutes exposure, the latter yielded 44 per cent

of good seedlings. Zea mays was found to be more resistant than Triticum or Hordeum but not so resistant as Lupinus or Pisum. After seventeen minutes exposure, 88 per cent of good seedlings of Zea mays were obtained; but from there on the percentage of germination fell rapidly. Thus after twenty minutes exposure, only 16 per cent of good seedlings were obtained; and, after twenty-five minutes exposure, only 4 per cent. Sinapis alba was slightly more resistant than Triticum. After a ten minutes exposure, 56 per cent of good seedlings were obtained. The foregoing tests and the following were made in a temperature of 20° to 22° C.

The second disinfectant employed was mercuric chloride. To prevent the adhesion of air-bubbles, the seeds were first dipped in 70 per cent alcohol, then thoroughly rinsed in water to remove the alcohol and finally placed in the mercuric chloride. The concentration of mercuric chloride employed varied from one gram of mercuric chloride in one thousand cc. of water to one gram in fifteen cc. of water. When Lupinus albus was immersed in a one to two hundred and fifty solution for fifty minutes, 100 per cent of good seedlings were still obtained. Beyond that concentration and length of immersion the percentage steadily decreased, until, at a concentration of one to fifteen for one hour and fifteen minutes, it had dropped to 52 per cent. For Pisum sativum a one to five hundred solution only was used. The yield of good seedlings, after a thirty minute immersion, was 88 per cent; and after a fifty minute immersion it was 64 per cent. Triticum vulgare was found to be much more sensitive to mercuric chloride than either Lupinus or Pisum. After a thirty-minute immersion in a one to five-hundred solution, only 52 per cent of good seedlings were obtained; while, after an immersion of ten minutes, the yield was 92 per cent. Hordeum vulgare was found to be extremely sensitive to the action of mercuric chloride. Even after an immersion of only two minutes in a one to one thousand solution, the yield of good seedlings was only 60 per cent. Zea mays was found to be more resistant to the action of mercuric chloride than Triticum. After an immersion of thirty minutes in a one to five hundred solution, the yield of good seedlings was 68 per cent; but in a one to two hundred and fifty solution, after the same length of time, the yield was only 20 per cent. Sinapis alba, after an immersion of ten minutes, in a one to one thousand solution yielded 76 per cent of good seedlings. When the concentration was increased to one to five hundred and the time to thirty minutes, the percentage dropped to eight. Before placing in the germinator, the seeds were thoroughly washed in several changes of water for about an hour.

The third disinfectant employed was peroxide of hydrogen. The commercial peroxide was used, and in no instance was it diluted. The length of time the seeds were immersed in it varied from ten minutes to nine hours. Lupinus albus, immersed for an hour, still yielded 100 per cent of good seedlings; when immersed for nine hours, the yield had dropped to 60 per cent. After an immersion of thirty minutes, Pisum sativum yielded 100 per cent, but after one hour, the yield was only 52 per cent. Triticum vulgare, after being immersed for an hour, yielded 84 per cent of good seedlings. Hordeum vulgare was found to be very sensitive to the action of the peroxide. After an immersion of only ten minutes, the yield was 48 per cent, but when immersed for an hour the yield fell to 12 per cent. After an immersion of nine hours, Zea mays yielded 76 per cent of good

seedlings. Sinapis alba was found to be more sensitive, since, after an hour's immersion, it yielded 64 per cent.

The fourth disinfectant employed was potassium dichromate. The concentrations employed varied from N/2 to N/1000; and the length of immersion varied from fifteen minutes to an hour. As in the case of the other disinfectants, the yield of Lupinus albus was relatively high, since the yield was still 84 per cent after an immersion of one hour in an N/2 solution. The highest concentration found practicable, in the case of Pisum sativum was N/50. After being immersed in this for an hour, the yield was 72 per cent. The same concentration could be used for Triticum vulgare. After a twenty-minute immersion, the yield was 88 per cent. Very poor results were obtained with Hordeum vulgare, since a twenty minute immersion in a solution as dilute as N/1000 yielded only 40 per cent of good seedlings. The concentration most useful in the case of Zea Mays was found to be N/10. The yield after an immersion of one hour, was still 56 per cent. N/25 was better adapted to Sinapis alba than any other concentration. After a twenty minute immersion, the yield of good seedlings was 88 per cent.

Ammonium persulphate was next used. The highest concentration used was one gram of the persulphate in three cc. of water the lowest concentration was one gram in one thousand cc. of water. The periods of immersion varied from thirty minutes to four hours and a half. The longer periods were used in the case of Lupinus albus only. The following concentrations were found to be best adapted to the different seeds; one to three for Lupinus albus, one to two hundred and fifty for Pisum sativum, one to one hundred for Triticum vulgare, one to fifty for Zea mays, and one to five hundred for Sinapis alba. When Lupinus alba was immersed for four hours and a half in a one to three solution, the yield of good seedlings was still 80 per cent. No higher concentration could be obtained, since that was a saturated solution. The lowest concentration, one to one thousand, was used for Hordeum vulgare only. When Hordeum vulgare was immersed for only ten minutes, the yield was only 28 per cent.

Since the halogens are good disinfectants, it was thought best to include at least one of them in these experiments. For this purpose bromine water was taken. The plain bromine water, used as a reagent in the chemical laboratory, was used. The bromine water was used full strength, as received from the chemical laboratory; and in dilutions as low as one part of bromine water to five thousand parts of water. The highest concentration was used for Lupinus albus only, and the lowest for Hordeum vulgare only. Lupinus albus immersed for an hour and forty minutes yielded 100 per cent of good seedlings. When immersed for six hours the yield was 44 per cent. Pisum sativum, immersed for ten minutes in a one to fifty solution, gave a yield of 64 per cent. The behavior of Triticum vulgare, at the different concentrations, was practically the same as Pisum sativum, except that in the lower concentrations Triticum gave a somewhat higher percentage of good seedlings Hordeum vulgare was so sensitive that it had to be thrown out. In a concentration of one to five thousand in which it was immersed for only ten minutes, the seeds were so much damaged that the yield of good seedlings was only 5 per cent.

Concentrations of one to fifty, one to one hundred, and one to two hundred and fifty were used for Zea mays. The lenght of immersion varied from ten to fifty minutes. The percentage yield varied from forty-four to seventy-two. Although higher concentrations were tried for Sinapis alba, one to five hundred was found to be the most suitable. After ten minutes immersion in this solution, the percentage of good seedlings was ninety-two; after fifty minutes immersion the percentage was sixty.

The last disinfectant used was formaldehyde gas. Forty per cent formaldehyde was put in an open vessel placed on a glass plate, on which the seeds were also placed. The whole was then covered with a bell-jar, the edge of which, where it came in contact with the glass plate, was given a coat of vaseline so as to make the chamber air-tight. Both dry seeds and seeds soaked for five minutes in water were used. The dry seeds of Lupinus albus had to be exposed for an hour and forty-five minutes before the germination percentage dropped; and then it had dropped only to ninety-six. When the soaked seeds had been exposed for forty-five minutes, the germination percentage dropped to ninetytwo. Beyond these periods, the vitality of the seeds steadily decreased, that of the soaked seeds becoming zero after seven hours, while the dry seeds retained their vitality an hour or two longer. The relative effect of the gas on dry and wet seeds is clearly brought out in the case of Pisum sativum. Dry seeds, exposed for fifteen minutes yielded 64 per cent of good seedlings; while the soaked seeds yielded only 28 per cent. To obtain a yield of 64 per cent in the case of the wet seeds, they could be exposed for two minutes only. After a twenty-five minute exposure of the wet seeds, only 16 per cent of good seedlings were obtained; while the dry seeds, exposed for an hour, still yielded 36 per cent. Triticum vulgare was found to be less sensitive to formaldehyde than Pisum sativum. After an exposure of fifteen minutes, the dry seeds yielded 92 per cent of good seedlings; while the soaked seeds still yielded 52 per cent. A ten-minute exposure of the wet seeds, also gave 92 per cent. Hordeum vulgare was found to be extremely sensitive to formaldehyde gas. A fifteen-minute exposure of the dry seeds and a five minute exposure of the wet seeds gave a germination percentage of only twelve. The dry seeds of Zea mays were considerably more resistant than Triticum; the soaked seeds only slightly so. Dry seeds, exposed for an hour, yielded 80 per cent of good seedlings, while wet seeds, exposed for thirty minutes, yielded only 36 per cent. The dry seeds of Sinapis alba could be exposed three times as long as the soaked seeds, and they still showed an equal vitality. Thus dry seeds exposed for forty-five minutes, and soaked seeds, exposed for fifteen minutes, both yielded 40 per cent of good seedlings. Dry seeds, exposed for thirty minutes, yielded 68 per cent; while wet seeds, exposed for the same length of time, yielded only 24 per cent.

In the first column of Table I are given the names of the seeds; in the second column the disinfectants; in the third column the concentrations best suited for the different kinds of seeds; in the fourth column the length of immersion of the seeds in the disinfectants which would still give a fair percentage of good seedlings; in the fifth column the germination percentage; and in the sixth column are given the results, as tested with the apparatus (see plate), of the action of the different disinfectants on the fungi and bacteria on the seeds. As can be seen by running over this column, only nine lots out of forty were found to be sterile, each lot being treated differently.

Table I.

Seed	Disinfectant	Concentration	Time	% of good Seedlings	Result
Lupinus albus	Cleaning Fluid	Full Strength	3 hrs. 45 Min.	68	Sterile
Pisum sativum	„ „	„ „	10 min.	68	2 Sp. Fungi
Triticum vulgare	„ „	„ „	3 „	76	1 „
Hordeum vulgare	„ „	„ „	4 „	76	1 „ „
Zea mays	„ „	„ „	15 „	76	1 „ „
Sinapis alba	„ „	„ „	8 „	72	Sterile
Lupinus albus	Mercuric chloride	1—15	1 hour	72	„
Pisum sativum	„ „	1—500	30 min.	72	Bacteria
Triticum vulgare	„ „	1—500	15 „	80	Sterile
Zea mays	„ „	1—500	20 „	68	„
Sinapis alba	„ „	1—1000	10 „	76	Bacteria
Lupinus albus	Hydrogen Peroxide	Full Strength (Com.)	7 hours	76	Sterile
Pisum sativum	„ „	„ „	45 minutes	76	Bacteria
Triticum vulgare	„ „	„ „	4 hours	80	Bacteria 1 Sp. Fungi
Zea mays	„ „	„ „	5 „	84	Sterile
Sinapis alba	„ „	„ „	45 minutes	64	„
Lupinus albus	Pot. Dichromate	N/2	1 hour	84	Bacteria
Pisum sativum	„ „	N/50	30 minutes	80	Bacteria 1 Sp. Fungi
Triticum vulgare	„ „	N/50	20 „	88	1 Sp. Fungi
Zea mays	„ „	N/10	30 „	72	1 „
Sinapis alba	„ „	N/25	20 „	96	Bacteria 1 Sp. Fungi
Lupinus albus	Ammonium Persulphate	1—3	4 hrs.	80	Bacteria
Pisum sativum	„ „	1—250	30 min.	80	„
Triticum vulgare	„ „	1—100	30 „	72	Bacteria 1 Sp. Fungi
Zea mays	„ „	1—50	40 „	68	Bacteria 1 Sp. Fungi
Sinapis alba	„ „	1—500	20 „	80	Bacteria
Lupinus albus	Bromine water	Full Strength	1—½ hrs.	92	Sterile
Pisum sativum	„ „	1—100	15 minutes	68	Bacteria
Triticum vulgare	„ „	1—50	30 „	72	1 Sp. Fungi
Zea mays	„ „	1—100	30 „	72	1 „ „
Sinapis alba	„ „ (Seeds dry)	1—500	30 „	80	1 „ „
Lupinus albus	Formaldehyde gas	40% Formal	4 hrs.	72	1 „ „
Pisum sativum	„ „	„ „	15 minutes	64	Bacteria
Triticum vulgare	„ „	„ „	30 „	72	„
Zea mays	„ „	„ „	30 „	64	1 Sp. Fungi
Sinapis alba	„ „ (Seeds wet)	„ „	30 „	68	Bacteria
Lupinus albus	Formaldehyde gas	„ „	2 hours	72	„
Pisum sativum	„ „	„ „	1 minute	72	„
Triticum vulgare	„ „	„ „	10 minutes	92	„
Zea mays	„ „	„ „	15 „	50	1 Sp. Fungi
Sinapis alba	„ „	„ „	5 „	80	Bacteria

I was not at all certain that the nine lots, which showed no contamination in the foregoing experiments, would show the same results again, if treated in a similar manner. For this purpose three lots of each were set up, treated as before and allowed to incubate. The results are given in table II.

Four of the nine failed to show up sterile three times in succession, while five remained sterile after incubation in all three tests. This means that apparently only five lots out of the original forty could be depended upon as being sterile when treated according to Table I. Of these five lots, three were Lupinus albus, and two were Sinapis alba.

Table II.

Seed	Disinfectant	Concentration	Time.	First Lot	Sterility Second Lot	Third Lot
Lupinus albus	Cleaning Fluid	Full Strength	3 hrs. 45 min.	Sterile	Sterile	Sterile
Sinapis alba	Cleaning Fluid	„ „	8 minutes	„	„	„
Lupinus albus	Mercuric Chloride	1—15	1 hour	„	Bacteria	Bacteria
Triticum vulgare	Mercuric Chloride	1—500	15 minutes	„	„	Sterile
Zea mays	Mercuric Chloride	1—500	20 minutes	„	Sterile	Fungus
Lupinus albus	Hydrogen Peroxide	Full Strength	7 hours	„	„	Sterile
Zea mays	Hydrogen Peroxide	„ „	5 hours	„	Bacteria	Fungus
Sinapis alba	Hydrogen Peroxide	„ „	45 minutes	„	Sterile	Sterile
Lupinus albus	Bromine water	„ „	1—½ hours	„	„	„

Since mercuric chloride is recognized as the most valuable desinfectant we have, it was thought best to test for sterility three lots each of the seeds used in these experiments. The length of time the seeds were immersed and the concentrations are those determined upon in the germination tests with mercuric chloride. By reference to Table I, the germination may be found. The results of these three tests are given in columns three, four and five, of Table III. None of these cultures showed growths of fungi or bacteria three times in succession and none were sterile three times in succession. The results in this case were rather disappointing, since they show that mercuric chloride cannot be depended upon to give sterile seeds in every case. The more so, since the aim was to test kinds of seeds fairly representative of all seeds likely to be used for laboratory purposes.

Table III.

Mercuric Chloride.

Seed	First Trial	Second Trial	Third Trial	Concentration and time.
Lupinus albus	Sterile	Bacteria	Bacteria	1—15 for 1 hr.
Pisum sativum	Bacteria	Sterile	Sterile	1—500 „ 30 min.
Triticum vulgare	Sterile	Bacteria	Sterile	1—500 „ 15 „
Zea mays	Sterile	Sterile	Fungus	1—500 „ 20 „
Sinapis alba	Bacteria	Bacteria	Sterile	1—1000 „ 10 „

Since the decisive failure to obtain sterile seeds, shown in Tables I, II and III, might reasonably be expected to make the technique appear open

to criticism, to say the least, it was thought advisable to set up a series of control cultures by means of the apparatus. After the apparatus had been sterilized in the autoclave, it was attached to the aspirator and air was drawn throught he filter plugs for at least five minutes to test them and at the same time to test the rubber connections. No seeds or disinfectant were placed in D. Water was then drawn from flask A into flask D. After this a small quantity of agar culture medium was drawn into flask D in the usual manner. Flask D was then taken out and set aside to incubate, as in the experimental cases. The idea was to test the apparatus by using it as nearly as possible in the same way as when seeds and disinfectants were present. As a matter of fact, I took less pains in the control experiments to see that the joints were perfectly tight than I did when I tested seeds. The fact that flask D was not opened during these control experiments cannot be held as an objection, since any chance contamination, when the seeds were placed in flask D, is reached by shaking up the disinfectant so as to thoroughly reach every part that might have become contaminated. If the chance spores were able to survive that, then it is manifestly impossible to kill those on the seeds, and the point is proven either way. The only place the disinfectant, when the seeds are treated, does not reach is the inside of the small glass tube connecting A and D. It was sterile when D was opened to admit the seeds and disinfectant and, during the brief period of time that D remained open, the mouth of the tube was directed downward, so no spores could drop in and none could be drawn in, since there was no draft into it. Besides, if any contamination could come from this source, in the case of the seeds, it would have appeared in the controls as well. Yet none of the controls showed any contamination. The conclusion seems irresistable that the contamination must have come from the seeds. Twenty controls were used.

Discussion.

The following three points will be considered in the discussion of results:

I. Some causes of failure to obtain seeds free from fungi and bacteria by disinfection methods.

II. Other methods that may be successful for obtaining seedlings free from fungi and bacteria.

III. Some reasons why much of the work on seed-sterilization is open to criticism.

I.

In view of the fact that out of six species of seed treated only two kinds, Lupinus albus and Pisum sativum, gave germinable seeds, free from fungi and bacteria, it is only reasonable to look for some cause or causes to explain this failure. The foregoing results are the more striking, since, out of the seven disinfectants used, only three were effective in securing seedlings free from bacteria and fungi, in the case of Lupinus albus and only two in the case of Sinapis alba. The cause of failure to obtain the desired result is at least three-fold. First, the condition of the contaminating organisms or their environment may make it impossible to destroy them, without destroying also the germ of the seed. Second ,the disinfectants, in a given case, may act merely as antiseptics, producing only apparently sterilized seeds. Third, the required concentration of the disinfectant and the required length of immersion may differ from those outlined in the preceding experiments.

Since conditions unfavorable for growth favor sporulation in bacteria, it is probable that the bacteria are found on the seeds as spores. As is well known, a bacterial spore is exceedingly more difficult to kill than a vegetating form. Add to this the protection the contaminating organisms enjoy from small fissures in the seed-coat, loose epidermal cells, etc., and you have a combination which aids the infecting organisms, but which gives little if any protection to the extremely sensitive seed-embryo. Novy and Waite's ('98) work on room disinfection conclusively shows that organisms, which may be quite readily destroyed when freely exposed to the desinfectant, will survive for hours, if treated in small masses, or if protected by small amounts of foreign substances. There is no bacterium known that can be immersed in a saturated solution of mercuric chloride for an hour and survive. Yet I found that bacteria survived that treatment when found on seeds. Protection of the organism can be the only explanation here. For this reason, the Fürbringer method might be useful in treating seeds. The value of this method lies in the preliminary use of alcohol. The alcohol dissolves and clears away from the seed-coat substances that interfere with the action of the disinfectant which follows it.

Not only may the contaminating organisms be protected by fissures in the seed-coat, debris, etc., but they may be found within the seed-coat. This is especially the case with fungi. To explain the overwintering of the rust on wheat, Eriksson formulated his Mycoplasm Theory. He claims that the fungus is present in the host cells as naked protoplasm, which could be distinguished from the protoplasm of the host by careful staining. Bolley and Pritchard ('95) say that what Eriksson saw in the cells of the seeds were the haustoria of the parasite. The Mycoplasm theory does not seem necessary to account for the fact. Bolley and Pritchard ('05) have shown that the mycelium of wheat rust penetrates the seed-coat and is thus carried over winter. Hannig ('08), working on Lolium temulentum, found a mycelium inside the seed-coat, growing over the aleurone layer. Freeman ('04) also working on Lolium temulentum, found that in some cases the mycelium had even penetrated the embryo. Whetzel ('06), working on beans, found that a fungus mycelium penetrated not only the pod but also the seed-coat and cotyledons. My failure to remove fungi from seeds must, it seems to me, be referred to a similar cause. It must be evident that when the fungus mycelium is within the seed-coat, as Hannig ('08) found in Lolium temulentum, or with the embryo as Whetzel ('06) found in the bean, or as Freeman found in the Lolium temulentum, it becomes hopeless to expect to kill it without at the same time killing the seed.

Of course the cases enumerated above were all instances of parasitic fungi. But, if they show anything, they show that mere superficial disinfection will not avail in all cases. However, a saprophyte may become a facultative parasite. It seems permissable to assume that many of the ordinary fungi found on seeds in the laboratory behave as facultative parasites. That they have penetrated the seed-coat to a greater or less depth cannot be doubted. I found a striking confirmation of this fact in my work with cleaning fluid. Lupinus albus and Sinapis alba were found to be free from infecting organisms of any kind. Pisum sativum, Triticum vulgare, Hordeum vulgare and Zea mays were found to be infected with fungi, after they had been treated with cleaning fluid till

nearly all of the seed-coat or fruit-coat had been destroyed. Pisum sativum and Zea mays even had two species of fungi growing on them after treatment with cleaning fluid. On none of these six lots did bacteria develope. These facts seem impossible of explanation, unless we assume that the fungi had penetrated the seeds.

The failure to obtain seeds free from contamination may also be due to the fact that the disinfectant acts as an antiseptic, which inhibis bacteria but allows them to develope when it is removed. This brings up the question: What is the difference between bactericidal and antiseptic action? Probably the correct answer is that it is merely a matter of degree. Sternberg ('01) makes the following distinction: "All disinfectants are also antiseptics, for agents which destroy the vitality of the bacteria of putrefaction arrest the putrefactive process: and these agents, in less amount than is required to completely sterilize, arrest growth and thus act as antiseptics. But all antiseptics are not germicides." Paul ('01) also says that inhibition, which occurs when a solution of one to one million of mercuric chloride is used, and sterilization are merely a matter of degree. Park (05)', in his work on "Pathogenic Bacteria and Protozoa", recognized the following four degrees: Attenuation, antisepsis, incomplete sterilization and disinfection. Antisepsis has not been recognized at all in work on seed sterilization, and not as much as it deserves in other work. It is very probable that in antisepsis lies the key to the problem of obtaining seeds free from bacteria and fungi, or rather, seeds on which the fungi and bacteria are inhibited from developing and multiplying. If by a dilute solution which will not harm the seeds, we can prevent the organisms from developing, the result, as far as the bacteria and fungi are concerned, will be the same as if the seeds were actually sterilized. Antisepsis is easily mistaken for disinfection, as Sternberg ('01) points out: "One to ten thousand solution of mercuric chloride destroyed the spores of B. anthracis and B. subtilis in two hours. More recent experiments show that failure to grow in culture solutions cannot be accepted as evidence of the destruction of vitality in the cases of spores exposed to the action of this agent, unless due precautions are taken to exclude the restraining influence of the small amount of mercuric chloride." Geppert ('89), working on B. anthracis, found that pieces of silk thread, soaked in a suspension of bacteria and then dried could be rendered apparently sterile by leaving them for ten minutes in a one to one thousand solution of mercuric chloride. Placed in bouillon and incubated for a sufficient length of time, no colonies developed. But, if similar threads were used in a similar manner, but treated with ammonium sulphide before being placed in the bouillon, Geppert found that abundant colonies developed. The ammonium sulphide reacts chemically with the mercuric chloride breaking up that powerful poison and forming the compounds ammonium chloride and mercurous sulphide. Several colonies were found to develope even in cases when the pieces of thread had been treated with mercuric chloride for half an hour. The objection that the silk threads afford protection to the bacteria might reasonably be raised here. But Geppert repeated his work with bacterial suspensions dried on cover-glasses, with similar results. These facts show that we are obliged to relegate many cases of so-called disinfection to antisepsis. My own work with mercuric chloride on seeds also seemed to indicate that the action was largely antiseptic. A marked difference was observed, when the seeds were merely in two or three changes of sterile water

and when they were washed for an hour in ten or twelve changes. Also a weak solution acting for a short time, but not so thoroughly washed off, was more effective in producing antisepsis than a strong solution, acting for a longer time and not so thoroughly washed off. At one time, when I had the pleasure of discussing the question of antisepsis with Dr. Novy, of the bacteriological laboratory of this university, he laid special emphasis on the size of the platinum loop with which inoculations were made from bacterial suspensions, to which a definite amount of mercuric chloride had been added as an antiseptic. He found, for instance, that enough mercuric chloride might be transferred on a 2mm. loop, when a tube of bouillon was inoculated from such a suspension, to inhibit the development of bacteria. A 1 mm. loop, on the other hand, might carry over insufficient mercuric chloride to produce such results. Or again, if, after thoroughly mixing the bouillon in the tube inoculated with a 2mm. loop, he inoculated a second tube of bouillon with a loopful of the first he would get a growth of bacteria in the second tube, while the first remained clear.

The last topic to be considered under the head of causes of failure to obtain seeds free from contaminating organisms is the matter of the required concentration of the disinfectant and the length of time the seeds should be immersed in it to produce the desired results. In other words: Is it, for instance, better to immerse a seed for a short time in a strong, or, for a longer time, in a weaker solution? There is always a possibility that this would lead us nowhere. Still there is also the possibility that concentrations and periods of immersion play a larger role in the relative effect on seeds and their infecting organisms than we now suppose. To determine this point is beyond the scope of the present paper. It would require a long series of experiments, in which only one disinfectant is used and the results of which are constantly checked by some such apparatus as was designed for the second series of experiments in this paper. Some work done in this laboratory on imbibition by seeds showed that about 80 percent of the water absorbed by the seed was absorbed during the first five minutes. Thus it would seem that the more rapid the action the better, provided it was stopped before it had reached the embryo. According to different authors, the concentration of mercuric chloride to be employed varied from 0.1 percent to 0.5 percent, and the recommended period of immersion from two or three minutes to half an hour. No rule can be laid down, since one species of seed is so much more resistant than another. Even different lots of the same kind of seed will vary so much that the treatment which will kill one sample will not injure another sample. For instance, Kehler (1904) immersed Triticum vulgare in a one to five hundred solution of mercuric chloride for half an hour and obtained 99.5 percent of good seedlings. When I tried to duplicate his results, I had only 52 percent of good seedlings. To complicate matters, the solution does not keep the same concentration during the entire experiment, since in some cases there is probably enough albumen on the seed-coats to combine with most of the available mercury, forming an insoluble albuminate. All of this goes to show how difficult it is, in work of this kind, to duplicate results. According to Novy and Waite (1895): „there is no chemic disinfectant which will invariably yield the same results regardless of the organism to be acted upon and the surroundings or environments of that organism". On comparing the results of other workers with each other and with my own, I am convinced that in seed sterilization this holds preeminently true. An

examination of the work of Pammel (1899) shows what part the seed-coat plays in the variability of the results obtained in two sets of experiments. Thickness, structure and composition, each adds something to the sum-total of the results obtained. In this connection, see note on Browns paper at the end of this paper, especially with reference to the extreme sensitiveness of Hordeum noted in the preceding pages. It is not possible here to enter into a full inquiry into and a complete discussion of the causes of the difference observed in the relative sensitiveness of the different seeds. To do that we should be obliged to devote too much space to the comparative histology of the seed-coats of the different seeds employed.

II.

There are three methods, other than disinfection, which should be discussed here. The first of these is excision of the embryo. This method has been employed especially by men working on endospermic respiration, respiration of the embryo, etc. It seems quite likely that sterile seedlings might be obtained by this method. But the conditions must be just right. In the first place, the embryo must be uninfected. When the fungus has penetrated it, excision of the embryo is naturally useless. Further it requires the most careful and delicate technique to obtain any results whatsoever by this method. How easily can an infecting germ be carried by the knife to the embryo, when the parts covering it are removed! If the seed-coat can be so thoroughly sterilized that there is no danger of infecting the knife, there can be no necessity for excising the embryo.

Another method is that adopted by Czapek (1896) for Zea mays. The dry grains were first polished by means of a dry, stiff brush, till no more scales came off; then they were thoroughly scrubbed for several minutes with warm water, soap and a brush; then they were rinsed in several changes of warm sterile water; and finally they were immersed for two or three minutes in a one percent solution of mercuric chloride. The seeds were then put in the germinator without first rinsing them. The main objection to this method is its limited applicability. It is obviously impossible to treat all seeds that way. Some seeds are too small, like Sinapis; or too light, like many of the gramineae; while the surface configuration, like that of Triticum, precludes such a procedure. Another objection was the comparatively large amount of mercuric chloride that was allowed to remain on the seeds, more than enough to cause antisepsis. No definite conclusion can be reached without a series of experiments with that end in view, as to the sterility or non-sterility of seeds thus treated.

A third method, employed by Harrison and Barlow (1907), may be called the selection method. About the only value I can see in this method is the elimination of infected seeds. The method they followed is as follows: One to three seeds were dropped in a test-tube containing about 3 cc. of boiling water. The tubes were immediately cooled and set aside. They were tilted so that the seeds were only half covered with water. Those tubes in which the water became cloudy, showing the presence of bacteria, were rejected. The seedlings in the tubes, in which the liquid remained clear, were then planted by means of sterile forceps in a sterile medium in Erlenmeyer flasks. These flasks were then set aside for four or five days. At the end of that time, any cultures showing contamination were again eliminated. The writers do not say what percentage of the original seeds were

left. It certainly can not have been large. It is also doubtful that the hot water acting for so short a time had any effect.

III.

In judging the value of previous work done on seed sterilization, it should be borne in mind that practically all that has been done was merely incidental. The author was, as a general rule, working on some problem for which it was advantageous to have sterile seedlings. Thus we find in papers on different pieces of work, tucked away here and there, a paragraph on seed sterilization. In practically all instances there can be no doubt that sufficient of the disinfectant remained to inhibit the development of such organisms as might be present. This point has been more fully discussed above. But what seems most remarkable is the lack of adequate proof that the seeds were actually sterile. Thus Nelson (1907) treating seed-potatoes for Oospora scabies, immersed them for an hour and a half in a one to one thousand solution of mercuric chloride, without any subsequent washing. They were allowed to dry and were then planted. Who shall say how long a goodly amount of mercuric chloride clung to the potatoes? The same objection holds in regard to Czapeks (1896) work. Steward (1908) claims that his seedlings were sterile at the end of fourteen or sixteen days. The only proof he offers is the fact that he inoculated a tube of bouillon with a little of the material scraped from a seedling with a platinum needle. The prevailing tendency seems to be a too great willingness to assume that the seedlings were sterile. Kehler (1904) obtained some striking results. His technique, however, is not above criticism. It seems to me extremely doubtful that a rubber tube can be sterilized by boiling for half an hour on three successive days the water in the flask to which it is attached. The more so when the flask is closed by means of a cotton plug around the tube entering it. This tube is withdrawn to above the water level during the boiling and again pushed down after it has been flamed. Finally the seeds are taken out of the receptacle and transferred to flasks of culture medium through the air. The apparatus should have been constructed in such a way as to prevent the seeds from coming in contact with the air at any time until the incubation was completed. And yet, for all that, Kehler claimed to have obtained sterile seeds in every case. Kehler does not speak of thorough and prolonged washing. That probably explains his results. Since Kehler mentions Geppert's work with ammonium sulphide, it seems almost inexplicable that he did not check up his own results by means of it. If he had done so and no organisms had developed, it would be conclusive proof of the correctness of his conclusions. Now, it seems to me, we are justified in doubting his conclusions.

Summary and Conclusions.

1. For certain physiological experiments, seeds free from bacteria and fungi are essential. Since there was no convincing evidence at hand that any of the methods used by others in disinfecting seeds, were absolutely reliable, this work was undertaken to supply, if possible, that evidence, or to prove that the methods generally employed are inade-

quate to furnish seeds free from contaminating organisms.

2. Lots of twenty-five each of the following species of seeds were used in the foregoing experiments: Lupinus albus, Pisum sativum, Triticum vulgare, Hordeum vulgare, Zea mays, and Sinapis alba. Similar lots in each case were treated for varying lengths of time with each of the following disinfectants: cleaning fluid, mercuric chloride, hydrogen peroxide, potassium dichromate, ammonium persulphate, bromine water, formaldehyde gas on dry seeds and formaldehyde gas on seeds soaked in water for five minutes. In the first series of experiments the efforts were directed toward determining the length of time each kind of seeds could be left in each one of the disinfectants, and still yield 70 percent or 80 percent of good seedlings. After this had been determined, a second series of experiments was used to determine the effect of the different disinfectants on the fungi, and bacteria, after the seeds had been in the disinfectant for such a length of time as would still permit a fair percentage of seeds to germinate.

3. The results obtained are rather strikingly opposed to those of other workers. When the action of the disinfectant was stopped at the point where still a fair percentage of the seeds were germinable, the results showed quite uniformly that the contaminating organisms had not been destroyed, except in a few instances noted below. Of the forty-eight lots tested, only two lots of Sinapis alba and three lots of Lupinus albus were free from bacteria and fungi. The only disinfectants which removed all contaminating organisms from these seeds were cleaning fluid and peroxide of hydrogen. Bromine water was also successful in the case of Lupinus albus. The foregoing experiments to determine the action of the disinfectants on fungi and bacteria were set up with a specially constructed apparatus, which prevented outside contamination.

4. In view of the results above, it was deemed best to set up twenty control preparations. They were set up in a manner similar to those above, except that no seeds or disinfectant were used. All of the control preparations were sterile at the end of two weeks, proving that the contamination, in the case of the seeds, must have come from the seeds themselves.

5. The foregoing work has convinced me that the results of formerly published work are open to criticism in at least two respects: No adequate proof

is given that the seeds are really free from contaminating organisms and no means are employed to remove the disinfectant so thoroughly that it can no longer act as an antiseptic. In view of the foregoing results, we are forced to conclude that the majority of cases of so-called disinfection were merely cases of antisepsis.

6. To antisepsis, and not to disinfection, we must probably look for practical results. It makes no difference in physiological experimentation whether a few dormant organisms cling to the seedlings or not. What does the harm is their active growth and multiplication. Absolute desinfection, which seems out of the question at present, is not essential.

Note.

Since the completion of the foregoing work, which was not published as soon as desired owing to unavoidable delays, several publications have appeared bearing on the same topic. Two of these are especially worthy of notice. Brown[1]) has shown that the seed-coats of Hordeum vulgare caerulescens are readily penetrated by mercuric chloride and some other agents, while sulphuric acid and copper sulphate affect it less quickly. He attributes this to a „selective action" of the seed-coat. I had noticed the same thing, but did not have an explanation for it. This perhaps accounts for the fact that I was unable to obtain seedlings from Hordeum, except when I worked with cleaning fluid.

The second paper is by Robinson[2]). The purpose of this work was to obtain „some definite knowledge of the effects produced by sterilization", thus admitting at the outset that there is undoubtedly a residual effect of sterilization that must be reckoned with. Thus the author takes practically the same ground that I have taken in the preceding paper.

Both leguminous and non-leguminous seeds were used. Fifty seeds of each of seven different kinds were used. The seeds were treated with the disinfectants, then rinsed several times in sterile, distilled water (a precaution many do not use) and germinated on moist, sterile filter paper in sterile Petri dishes. After the seeds had been in the Petri dishes for serveal days, platings were made on beef agar from the seeds.

From the author's paper I infer that he did not subject his seeds to as long and thorough washing as I did. That is the only way I can explain the difference between his results and mine. For instance, he found that wheat and corn were sterile after treating them for one hour with commercial hydrogen peroxide. In my work I found that they were not sterile even after treating wheat four hours and corn five hours. The germination percentage was practically the same in his case and mine. The author himself calls attention to the fact that enough disinfectant may adhere to the seeds to cause antisepsis. Thus, some seeds of pea, wheat and radish were treated for thirty minutes with a 0.5 percent solution of mercuric chloride, they were then washed three times and the third wash water was used for plating Bacillus sub-

[1]) Proceed. of the Roy. Soc. London. Vol. 81. Ser. B. p. 82.
[2]) U. S. Dept. of Ag. Bur. of Plt. Ind. Circ. No. 67.

tilis. At the end of the period of incubation the plates were sterile, showing that enough mercuric chloride remained on the seeds after two washings to make the third wash water so toxic that it inhibited the growth of Bacillus subtilis. Formaldehyde and hydrogen peroxide showed the same results, unless used in a very weak solution and for a very short time.

The author also found that air-bubbles on or in the seeds interfered with the action of the disinfectant. To overcome this difficulty he used a vacuum-pump. The results are described as „good but not perfect". I found treating them for a moment with 70 percent alcohol satisfactory. If the vacuum-pump is used, the disinfectant is apt to penetrate too deeply to be readily removed.

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